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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/10, C12P 21/22		A1	(11) International Publication Number: WO 91/15573
			(43) International Publication Date: 17 October 1991 (17.10.91)
<p>(21) International Application Number: PCT/US91/02296 (22) International Filing Date: 2 April 1991 (02.04.91)</p> <p>(30) Priority data: 504,171 3 April 1990 (03.04.90) US</p> <p>(71) Applicant: SOUTHWEST FOUNDATION FOR BIOMEDICAL RESEARCH [US/US]; 7620 N.W. Loop 410 at Military Drive, P.O. Box 28147, San Antonio, TX 78284 (US).</p> <p>(72) Inventors: BURK, Kenneth, H. ; 15630 Cloud Top, San Antonio, TX 78248 (US). JACOB, James, R. ; 2666 Lakewood Avenue, Hamberg, NY 14075 (US). LANFORD, Robert, E. ; 1902 Hillingway, San Antonio, TX 78248 (US).</p>			(74) Agent: DEHLINGER, Peter, J.; Dehlinger & Swiss, P.O. Box 60850, Palo Alto, CA 94306 (US).
			(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).
<p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>			
<p>(54) Title: AN IMMORTALIZED PRIMATE HEPATOCYTE CELL LINE</p>			
<p>(57) Abstract</p> <p>An immortalized primate hepatocyte cell line which produces normal liver secretory proteins, and which is able to support replication by human hepatotropic viruses is disclosed. The cell line is useful in producing hepatotropic viruses, such as hepatitis C virus, and for screening drug compounds for ability to inhibit the growth of such viruses. The cell line is also useful as a source of liver-specific protein products.</p>			
<p>Western blot analysis showing protein expression in immortalized primate hepatocyte cell line. Lanes include Total, ApoE, ApoA1, Pre-alb, β_2 micro., CRP, Plasm., C4, Trans., α1AT, and Alb. Labels on the left indicate bands for Trans., Alb, α-1-AT, ApoE, and ApoA1.</p>			

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AN IMMORTALIZED PRIMATE HEPATOCYTE CELL LINE10 1. Field of the Invention

The present invention relates to an immortalized primate hepatocyte cell line, and to uses of the cell line for culturing hepatotropic viruses and for producing hepatocyte secretory products.

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2. References

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3. Background of the Invention

Hepatocytes which can be maintained successfully in culture, in a differentiated, infectable state, would be advantageous in studying and producing human hepatotropic viruses, such as hepatitis A virus (HAV), hepatitis B virus (HBV), enterically transmitted Non-A, Non-B hepatitis virus, also now known as hepatitis E virus (HEV), and 15 parenterally transmitted Non-A, Non-B hepatitis virus, also now known as hepatitis C virus (HCV).

Such hepatocytes would allow screening of drug compounds, for effectiveness against virus growth. The cells could also provide a source of intact, active or 20 attenuated virus particles, for direct production of the virus, or for use in obtaining mature virus proteins or peptide fragments from isolated virus particles.

Recently, the inventors have described cell culture conditions which permit long-term growth of primary primate 25 hepatocytes in culture, in a differentiated state (Lanford, 1989). The cultured, primary hepatocytes produce several liver-specific secretory proteins, such as albumin, α -1-antitrypsin, complement C'4, fibrinogen, apolipoproteins A-1 and E, transferrin, and/or plasminogen for culture 30 periods of 100 days or more. These cells are infectable by HCV and are able to support replication of the virus in culture. The use of the cultured cells to produce intact

mature HCV virus particles is reported in the companion PCT patent application "Purified Non-A, Non-B Hepatitis Virus".

One limitation of primary, cultured primate hepatocytes, however, is that the differentiated cells lose many 5 of their liver-specific functions within 3-4 weeks of infection. Therefore, new primate hepatocytes must be continually obtained, prepared for cell culture, and infected with virus when growing hepatotropic virus in culture. Another limitation of the cells is the problem of 10 maintaining controlled, uniform cell properties for virus growth when the hepatocytes are continually being replaced by new cells.

4. Summary of the Invention

15 One general object of the invention is to provide an immortalized primate hepatocyte cell line able to support the growth of hepatotropic viruses over extended culture periods.

The invention includes, in one aspect, an immortalized 20 primate hepatocyte cell line whose cells are characterized by (a) an oncogene integrated in the cellular genome; (b) synthesis and secretion of normal liver secretory proteins, and (c) ability to support replication of human hepatotropic viruses. More specifically, the cells are charac-25 terized by stable secretion in culture of several, i.e., at least three of the following liver secretory products: albumin, α -1-antitrypsin, complement C'4, fibrinogen, apolipoproteins A-1 and E, transferrin, and plasminogen.

In one embodiment, the cell line is derived from 30 chimpanzee or human hepatocytes, and the cells are infectable with and support replication of hepatitis C

virus. The oncogene in one preferred immortalized chimpanzee and marmoset hepatocyte cell line is the SV40 large T antigen.

In another embodiment, the cell line is derived from 5 baboon hepatocytes, and the oncogene is a combination of adenovirus E1A and cellular myc oncogenes.

The invention also includes a method of producing hepatotropic virus particles. The immortalized primate hepatocyte cell line, infected with the virus, is cultured 10 under conditions which maintain the liver-specific functions of the cell, and virus particles produced by the cells are harvested from the culture medium.

In one preferred embodiment, the cell line is derived from human or chimpanzee hepatocytes, and the virus is 15 hepatitis C virus (HCV).

Also disclosed is a method for screening compounds for the ability to inhibit growth of an hepatotropic virus. In this method, the immortalized primate hepatocyte cell line from above is cultured under conditions which maintain the 20 liver-specific functions of the cell. After exposing the cell to virus, cultured cells are exposed to a test compound for a selected period of time, and the cells are assayed for the amount of virus present, typically by assaying for the presence of viral nucleic acid.

25 In still another embodiment, the invention includes a method of producing liver secretory proteins, such as albumin, α -1-antitrypsin, complement C'4, fibrinogen, apolipoproteins A-1 and E, transferrin, and plasminogen, by isolating the desired protein from the above immortalized 30 cells in culture.

These and other objects and features of the present invention will become more readily apparent when the

following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

5 Figures 1A-1D are phase contrast photomicrographs of immortalized chimpanzee hepatocyte (CHMP) cells with predominant morphologies characterized by (1A) compact cells exhibiting minimal cytoplasm, (1B) spindle-like cells with cytoplasmic extensions, (1C) flattened cells
10 exhibiting a granular and enlarged cytoplasmic area, and (1D) cuboidal cells exhibiting a morphology resembling normal primary hepatocytes in culture;

Figures 2A-2D are phase contrast photomicrographs of immortalized baboon hepatocyte cells immortalized with (2A)
15 U19 retrovirus expressing SV40 large T antigen, (2B) a plasmid encoding both SV40 large and small T antigens, (1C) plasmids containing both the myc and ras oncogenes, and (1D) plasmids containing both the E1A and myc oncogenes;

Figure 3 shows electrophoretic patterns of total
20 proteins secreted by the CHMP cell lines indicated at the top of the figure, where the numbers at the left in the figure indicate molecular weights (in kilodaltons) of known marker proteins;

Figures 4A and 4B are gel electrophoretic patterns of
25 proteins secreted by normal chimpanzee hepatocytes (4A) and CHMP 1.20 cells (4B), and prepared by immunoprecipitation of total secreted proteins with antibodies specific against the proteins indicated at the top in the figures, followed by electrophoresis of the precipitated proteins;

30 Figure 5 shows gel electrophoresis patterns of proteins secreted by immortalized baboon hepatocytes, and

prepared by immunoprecipitation of total secreted proteins with antibodies specific against the proteins indicated at the top in the figure, followed by electrophoresis of the precipitated proteins;

5 Figure 6 shows electrophoretic patterns of PCR (polymerase chain reaction) products of cellular RNA from various immortalized chimpanzee hepatocyte cell lines (CU cell lines) derived from an HCV-infected chimpanzee (lanes 1-8), and from chimpanzee liver RNA during the acute phase
10 of HCV infection (lane 9);

Figure 7 shows electrophoretic patterns of PCR products of RNA from various CHMP cell lines infected in vitro with HCV (lanes 1-12 and 14), from the inoculum used to infect the cells (lane 13), from chimpanzee liver RNA
15 during the acute phase of HCV infection (lanes 15 and 18), and from an HCV cloned fragment; and

Figure 8 shows electrophoretic patterns of PCR products of RNA from various HAV-infected immortalized cells derived from marmoset hepatocytes (lanes 6 and 7), from
20 control HAV inoculum (lanes 10 and 11), and from PCR positive controls (lanes 12 and 13).

Detailed Description of the Invention

25 I. Immortalization Procedures

This section describes methods for producing an immortalized primate, hepatocyte cell line whose cells have the following characteristics:

- (a) an oncogene integrated in the cellular genome;
- 30 (b) secretion in culture of several (at least three) liver-specific hepatocyte secretory proteins, such as albumin, α -1-antitrypsin, complement C'4, fibrino-

gen, apolipoproteins A-1 and E, transferrin, and plasminogen; and

(c) ability to support replication of human hepatotropic viruses, such as hepatitis A virus (HAV),
5 hepatitis B virus (HBV), hepatitis E virus (HEV), and hepatitis C virus (HCV).

A. Cultured primary hepatocytes

The immortalized cell line is derived from, i.e.,
10 obtained from primary primate hepatocytes which are cultured under conditions which maintain liver-specific functions, particularly the ability to produce and secrete liver-specific proteins, for periods of up to 100 days or more in culture. Methods for preparing primary primate
15 hepatocytes for culture, and culture medium conditions effective to preserve liver-specific functions for extended periods in culture have been described by the inventors (Lanford, 1989). Briefly, liver tissue obtained by liver biopsy from a human, chimpanzee, baboon, marmoset or other
20 primate, is perfused and hepatocytes are dislodged by treatment with collagenase. The cells are washed several times, then plated on culture plates at a density of about
5 x 10⁵ to 5 x 10⁶ cells per 60 mm plate.

The hepatocytes are maintained in serum-free medium
25 (SFM) which has been specifically designed to allow the cells to grow in culture in a liver-differentiated form, as evidenced by the continued production and secretion in culture of liver-specific proteins. One preferred culture medium is composed of Williams' medium E (WME) supplemented
30 with 10 mM HEPES, pH 7.4, 50 ug gentamycin, and the following supplements: EGF (epidermal growth factor), insulin, glucagon, BSA (bovine serum albumin), soybean

lipids, linoleic acid, hydrocortisone, selenium, cholera toxin, LGF (liver growth factor, a glysyl-histidyl-lysine tripeptide), ECFS (endothelial cell growth supplement), transferrin, ethanolamine, prolactin, somatotropin, and TRF 5 (thyrotropin-releasing factor), in the proportions given in Example 1. The sources of these materials are given elsewhere (Lanford).

The cells are maintained in the SFM under standard cell culture conditions. The medium is changed, e.g., 24 10 hours after isolation and every 48 hours thereafter, during the culture period. For some primate hepatocytes, such as derived from chimpanzee liver, the cells appear to undergo 2-4 rounds of replication in the first several days of culture, e.g., within 7-10 days, and thereafter continue to 15 function as liver-specific cells in culture, but without appreciable signs of cell replication. In other primate hepatocytes, such as derived from baboon and marmoset liver, the primary cells also undergo 2-4 rounds of replication in culture, but thereafter, continued cell 20 replication is observed from foci in the original culture plate, and these foci undergo replication until they grow to a monolayer on the plate. These cells in the monolayer are still differentiated hepatocytes, as evidenced by continued production and secretion of liver-specific 25 proteins. This distinction between different types of cultured primary primate hepatocytes is mentioned because the strategy for selecting immortalized cells may vary according to the replication behavior of the cells during the immortalization step.

30 A variety of methods are available to confirm that the cultured primary cells are producing and synthesizing liver-specific secretory proteins. Two of these methods,

involving analysis of total culture medium by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoprecipitation with antibodies against liver-specific proteins, are described in Example 3, with reference to detection of liver-specific products produced by the immortalized liver cells of the invention. Typically, the primary cells will produce serum albumin, α -1 antitrypsin, complement C'4 apolipoprotein E and A-1, fibrinogen, plasminogen, transferrin, in combinations including at least three of these proteins.

B. Cell Immortalization

The primary cells from above are immortalized by integrating into the cell genome, an oncogene effective to inhibit the normal cell-replication control mechanism(s) of the primary cells in culture. The sources of a variety of oncogenes, and their mechanism of action in releasing normal cell replication constraints have been reviewed (Bishop). Typically, the oncogenes are isolated from viruses which have picked up an oncogene from a mammalian host genome, and where the oncogene exists in a mutated form which produces an activated, i.e., unregulated gene product effective to overcome a cell's normal growth-inhibition mechanism(s). Table 1 below lists several of the oncogenes which have been described to date in the literature.

Table 1

	Abbreviation	Virus
5	arc	Rous Sarcoma Virus (Chicken)
	yes	Y73 Sarcoma Virus (Chicken)
	fps	Fujinami (St. Feline) Sarcoma Virus
	abl	Abelson Marine Leukemia Virus (Mouse)
	ros	Rochester-2 Sarcoma Virus (Chicken)
10	fgr	Gardner-Rasheed Feline Sarcoma Virus (Cat)
	erba	Avian Erythroblastosis Virus (Chicken)
	fms	McDonough Feline Sarcoma Virus (Cat)
	mos	Moloney Murine Sarcoma Virus (Mouse)
	raf	3611 Murine Sarcoma+ Virus (Mouse)
15	Ha-ras-1	Harvey Murine Sarcoma Vi- rus (Rat) (Balb/c mouse; 2 loci)
	Ki-ras 2	Kirsten Murine Sarcoma Virus (Rat)
	Ki-ras 1	Kirsten Murine Sarcoma Virus (Rat)
	myc	Avian MC29 Myelocytomato- sis Virus (Chicken)
	myt	Avian Myelo Blastomas (Chicken)

Abbreviation	Virus
fos	FBJ Osteosarcoma Virus (Mouse)
ski	Avian SKV T10 Virus (Chicken)
rel	Reticuleondotheliosis Virus (Turkey)
sis	Simian Sarcoma Virus (Woolly Monkey)
5 N-myc	Neuroblastomas (Human)
N-ras	Neuroblastoma, Leukemia Sarcoma Virus (Human)
Blym	Bursal Lymphomas (Chicken)
man	Mammary Carcinoma (Human)
neu	Neuro, Blioblastoma (Rat)
10 ertA1	Chicken AEV (Chicken)
ra-ras	Rasheed Sarcoma Virus (Rat)
mnt-myc	Carcinoma Virus MH2 (Chicken)
myc	Myelocytomatosis CK10 (Chicken)
myb-ets	Avian myeloblastosis/ erythroblastosis Virus E26 (Chicken)

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The oncogenes can be placed recombinantly in plasmids which are capable of infecting a suitable host cell.

20 Alternatively, the oncogenes can be placed in a suitable virus vector, such as a retrovirus (Miller) vector which is

capable of infecting the host cell, with constitutive production of the oncogene product. A variety of plasmid and virus constructs which carry oncogenes and which are capable of introduction into mammalian cells in culture 5 have been reported, and are available, either from public depositories such as the American Type Culture Collection (Rocklawn, MD), or by using recombinant plasmid techniques to form plasmid constructs described in the literature. In the method detailed in Example 1 for the immortalization of 10 chimpanzee primary hepatocytes, and in Example 6 for immortalization of marmoset hepatocytes, the oncogene for the SV40 large T antigen, which is defective for binding to the SV40 origin of DNA replication, was used for immortalization. The oncogene was introduced by way of a U19 15 retrovirus, whose genome was designed for constitutive expression of the T antigen. The retrovirus can be grown in and harvested from the culture medium of a suitable host, such as the U19-5 cell line described in Example 1.

In the method detailed in Example 5 for the immortalization 20 of baboon hepatocytes, four different oncogenes or oncogene combinations were tested for ability to immortalize the primary cells. In the first method, the cells were infected by the U19 retrovirus containing the oncogene for SV40 large T antigen, described above. In the second- 25 fourth method, the cells were exposed to various plasmid constructs containing either ras, myc, or E1A oncogenes. The second method involved the pSV3neo plasmid containing both large and small SV40 T antigen oncogenes, and a neomycin resistance gene. The third method involved a 30 combination of plasmid pSVC-myc-1, which contains the myc oncogene, and plasmid PUJ EJ 6.6, which contains the ras oncogene. The fourth method involved a combination of the

plasmid p1Aneo, which contains the E1A oncogene, and the above plasmid pSVc-myc-1. The last-mentioned method produced cells with highly differentiated morphology, as noted in Example 5, while the first three methods produced 5 cells which appear more transformed and less differentiated.

The oncogene may be introduced into the primary cells using a variety of vectors. Where the oncogene is contained in an infective viral vector, such as the U19 10 retrovirus noted above, the oncogene is introduced by viral infection, typically by exposing the cells to the virus for a period of several hours, then washing the cells to remove free virus. Where the oncogene is carried on a plasmid, standard methods for introducing plasmids into mammalian 15 cells may be used. These include electroporation, and plasmid uptake in the presence of CaCl₂ or lipofection (Felgner). The latter method is preferred, since it allows introduction of plasmids with reasonable efficiency and little cell disruption. Details of retrovirus infection 20 for immortalization of chimpanzee and marmoset hepatocytes are given in Examples 2 and 6, respectively. Immortalization of baboon hepatocytes with plasmids in the presence of lipofection are given in Example 5.

To introduce the oncogene, the hepatocytes are plated 25 at a subconfluent level which allows several rounds of replication after the oncogene vector is introduced. The cells are exposed to the oncogene vector for a period of typically several hours, after which the cells are washed to remove free vector, and then cultured in SFM. As noted 30 above, primate cells will undergo 2-4 rounds of replication in the first 7-10 days under the culture conditions

described above. During this period, the culture medium is changed every 1-3 days.

At the end of the period of initial replication, e.g., after 7-10 days in culture, the culture will consist of 5 both immortalized and non-immortalized hepatocytes. At this point, it may be necessary to expose the cells to a selection pressure which allows selective proliferation of the immortalized cells. To this end, the oncogene vector is provided with a selective marker, such as an antibiotic 10 resistance gene, which allows growth selection in the presence of the antibiotic. In the case of chimpanzee hepatocytes, which tend to cease replication at the end of initial 2-4 rounds of replication, it may not be necessary to subject the cells to antibiotic selection, since the 15 immortalized cells will gradually take over the remaining, non-replicating primary cells. Here the cells may be allowed to expand in culture for a period of 3-5 weeks, until colony outgrowths, represented by immortalized cell colonies, are observed. Alternatively, an antibiotic may 20 be added after the initial phase of replication, to insure complete removal of non-immortalized cells from the culture.

In the case of primate hepatocytes, such as baboon or marmoset hepatocytes, which show continued growth of 25 primary cells from foci, after the initial round of replication, the cells are first grown in the absence of antibiotic (or at a low level of antibiotic) for 7-10 days, or until the initial 2-4 rounds of replication occur. At this point, the cells are then exposed to the antibiotic, 30 at a concentration sufficient to selectively kill the non-immortalized primary cells (which do contain the gene for antibiotic resistance). The cells are then cultured

further in the presence of the antibiotic until colony outgrowths are observed. The cells are not exposed to antibiotic during the initial rounds of replication to prevent premature killing of cells before expression of the 5 antibiotic resistance gene in the oncogene vector can occur, and to reduce toxicity to the cells in culture by cellular products released from dying cells.

After colony outgrowths of immortalized cells are obtained, the cells are again disrupted, e.g., by collagenase/dispase treatment. The cells are then resuspended in a suitable attachment medium, such as WME (Williams Medium E) supplemented with 5% fetal bovine serum (FBS), and replated at low density. After 3 hours, the cultures are changed to SFM to allow single-colony outgrowths to be 15 isolated. The resultant immortalized colonies are then characterized, to identify cell lines which (a) retain their liver-cell differentiation, as evidenced by production of liver-specific proteins, and (b) are infectable with hepatotropic viruses. These characterization methods 20 are discussed in Section II.

II. Characterization of the Immortalized Cells

A. Morphology

The immortalized cells may have one of several 25 distinguishable morphologies which depend on the primate species from which the hepatocytes are derived, and the oncogene(s) used in the immortalization method.

Figures 1A-1D show four of the distinguishable morphologies which have been observed in immortalized 30 chimpanzee hepatocytes, produced by U19 retrovirus immortalization. The Figure 1A cells are compact cells which exhibit minimal cytoplasm. Cells line having this general

morphology are represented by the selected cell lines designated CHMP 5.13, CHMP 1.05, CHMP 2.01, CHMP 2.06, and CHMP 3.12. The Figure 1B cells are spindle-like cells with cytoplasmic extensions. Cells having this morphology are 5 represented by the cell line designated CHMP 5.01.

The Figure 1C cells are flattened cells exhibiting a granular and enlarged cytoplasmic area. Representative cell lines include the one designated CHMP 5.03. The fourth cell morphology type has cuboidal cells which 10 resemble normal primary cultured hepatocytes morphologically, as represented by cell line CHMP 5.04.

The variation in cell morphology which can be produced by different oncogenes is illustrated by the four morphology types seen in Figures 2A-2D, which show photomicrographs 15 of baboon hepatocytes immortalized with a retrovirus (2A), or one or more oncogene-containing plasmids. Specifically, the hepatocyte cells immortalized with (2A) U19 retrovirus expressing SV40 large T antigen, (2B) a plasmid encoding both SV40 large and small T antigens, (1C) plasmids 20 containing both the myc and ras oncogenes, and (1D) plasmids containing both the E1A and myc oncogenes. As seen, the ^{^T}T cell line (Figure 2D) has a highly differentiated morphology, while the other cell lines appear more transformed and less differentiated.

25

B. Secretory Proteins

According to an important aspect of the invention, the immortalized primate hepatocytes cells retain hepatocyte differentiation, as evidenced by the ability of the cells 30 to produce several, i.e., at least three, liver-specific secretory proteins, such as albumin, α -1-antitrypsin, complement C'4, fibrinogen, apolipoproteins A-1 and E,

transferrin, and plasminogen, but also including minor liver-specific proteins, such as C-reactive protein, apolipoproteins a-II, apolipoproteins C2 and C3. Preferably, the cells produce the secretory proteins albumin, α -1 antitrypsin, one or both apolipoproteins A-1 and E, complement C'4, and one or both clotting factors plasminogen and fibrinogen.

Preferably, the secretory proteins are made in approximate relative proportion to their concentrations in the serum. That is, the major liver secretory proteins in the plasma, such as albumin, α -1 antitrypsin, and transferrin, are the major secretory proteins produced by the immortalized cells, and the minor proteins in the plasma are the minor ones produced in the cell culture.

The presence of liver-specific secretory proteins in the cell culture medium can be confirmed by a variety of methods. In one, described in Example 3, hepatocyte cultures representing compact cell morphology (CHMP 5.13, CHMP 1.05, CHMP 2.01, CHMP 2.06, and CHMP 3.11), flat cell morphology (CHMP 5.13), and cuboidal cell morphology (CHMP 5.04) were incubated with [³⁵S] methionine, and radiolabeled proteins from culture medium were fractionated by SDS-PAGE. Figure 3 shows autoradiographs of culture-medium proteins from the several CHMP cell lines. The major proteins detected by this analysis had molecular sizes corresponding to albumin (67kd) and alpha-1-antitrypsin (54kd). The size markers (molecular weight given in kilodaltons) were phosphorylase B (93kd), serum albumin (67kd), ovalbumin (43kd), carbonic anhydrase (30kd), trypsin inhibitor (20kd), and lysozyme (14kd). Details of the method are given in Example 3.

Secretory proteins in the culture medium can be positively identified by immunoprecipitation methods, such as detailed in Example 3. Here culture medium containing radiolabeled proteins are reacted with immobilized antibodies specific against one of a number of different primate, e.g., human, serum proteins. The immunospecifically bound proteins are then released from the antibodies and fractionated by SDS-PAGE, as detailed in Example 3. The identity of the immunoprecipitated proteins is indicated at the top in Figures 4A and 4B. The profile of secretory proteins obtained with primary hepatocytes (Figure 4A) was very similar to that observed for the CHMP 1.20 cell line (Figure 4B), demonstrating the retention of liver-specific functions, i.e., a highly differentiated state, after immortalization.

A similar immunoprecipitation method was used to determine the identity of secretory proteins expressed in several CHMP cell lines. The results are summarized in Table 2. The "+" symbol in the table means normal expression, i.e., expression comparable to that observed in primary cultures; the "*" symbol, low expression; the "--" symbol, no expression; and "NT", not tested.

Several of the lines, as exemplified by CHMP 1.20, expressed a majority of the plasma proteins investigated. The level of expression of the apolipoproteins A-1 and E in many of the cell lines was similar to that observed in primary cultures, although β_2 -microglobulin and prealbumin expression levels were elevated.

Complement C'4 was detected in all the cell lines; however, one or two of the associated chains (alpha or gamma) were not detected by precipitation with the respective antibody. C-reactive protein was not detected in any

cell line. CHMP 5.01 was deficient in albumin expression although it still expressed several other plasma proteins including apo E. The one cell line is clearly deficient in differentiated liver cell functions is CHMP 2.03, which 5 produces only α -1 antitrypsin among liver-specific proteins.

No attempt was made to investigate the possible intracellular accumulation of plasma proteins which may have occurred due to the transformed state of the culture 10 in question. Unlike the established human hepatocellular carcinoma cell lines, no α -fetoprotein has been detected in any primate hepatocyte cell line examined to date.

Similar protein-identification studies were carried out with immortalized baboon cells, as described in Example 15 6. Figure 5 shows in lane A, total secreted proteins present in the culture medium, after fractionated by SDS-PAGE. The identification of several liver secretory proteins, based on migration distance on the gel, is shown at the left of lane 1. Immunoprecipitation of the radiola- 20 beled culture proteins, and subsequent fractionation by SDS-PAGE, indicates the relative amounts of various proteins, including apolipoproteins E and A-1, pre-albumin, plasminogen, complement C'4, transferrin, α -1 antitrypsin, and albumin, as seen.

25

C. Oncogene Expression

The immortalized cells can be assayed for expression of the oncogene, by similar immunoprecipitation methods for detecting oncogene products in the culture medium. For 30 example, the large T antigen product of the U19 oncogene used to immortalize chimpanzee hepatocytes was identified by immunoprecipitation of the T antigen in each of the

20

immortalized CHMP cells reported in Table 2, as indicated at the right in the table.

Table 2

	<u>Primaries</u>	1.20	1.33	2.02	2.03	2.05	3.01	4.03	4.07	5.01
Albumn	+	+	+	-	+	+	+	+	+	-
$\alpha_1\alpha$ Tryp	+	+	+	+	+	+	+	+	+	+
apo A-1	+	+	*	-	*	-	+	+	-	-
apo E	+	+	+	-	*	*	+	+	+	+
β_2 -micro	+	+	+	+	+	+	+	+	+	+
C'4	+	+	+	*	+	+	+	+	+	+
CRP	+	-	-	-	-	-	-	-	-	-
Fibrinogen	+	+	+	-	+	-	+	+	-	-
Plsmgn	+	-	-	*	-	-	-	-	*	-
PreAlb	+	+	*	*	-	-	+	*	*	*
Transfrn	+	+	*	*	-	*	+	+	+	+
AFP	-	-	-	-	-	-	-	-	-	-
T-ag	N.T.	+	+	+	+	+	+	+	+	+

Immunoprecipitation of liver specific plasma proteins:

+ = Normal Expression

* = Low Expression

- = No Expression

N.T. = Not Tested

Several of the lines, as exemplified by CHMP 1.20, expressed a majority of the plasma proteins investigated. The level of expression of the apolipoproteins A-1 and E in many of the cell lines was similar to that observed in primary cultures, although β_2 -microglobulin and prealbumin expression levels were elevated.

Complement C'4 was detected in all the cell lines; however, one or two of the associated chains (alpha or gamma) were not detected by precipitation with the respective antibody. C-reactive protein was not detected in any cell line. CHMP 5.01 was deficient in albumin expression although it still expressed several other plasma proteins including apo E. The one cell line that is clearly deficient in differentiated liver cell functions is CHMP 2.03, which produces only α -1 antitrypsin among liver-specific proteins.

No attempt was made to investigate the possible intracellular accumulation of plasma proteins which may have occurred due to the transformed state of the culture in question. Unlike the established human hepatocellular carcinoma cell lines, no α -fetoprotein has been detected in any primate hepatocyte cell line examined to date.

Alternatively, the presence of the oncogene in the immortalized cells can be determined directly, by PCR amplification of the oncogene sequences in a cell genomic digest. This approach was used to confirm the presence of the oncogene in the cell genome in a number of immortalized cell lines.

30 D.. Infectivity by Hepatotropic Viruses

Another important property of the immortalized primate hepatocytes of the present invention is the ability to

support replication of human hepatotropic viruses. By this is meant that the cell line can be infected with an active hepatitis virus, and the virus can replicate in the cultured cells, with the production of mature virus particles, 5 preferably in the culture medium.

One hepatitis virus of particular interest, because of its importance as a blood-borne pathogen, is HCV. The ability of CHMP cells to support HCV infection is demonstrated from the studies reported in Example 4. Here CHMP cells 10 (prepared by immortalization of non-infected chimpanzee hepatocytes) and CU cells (similarly prepared from HCV-infected chimpanzee hepatocytes) were both inoculated with chimpanzee plasma known to contain HCV, as detailed in Example 4. On the 11th day after infection, the cultures 15 were harvested for analysis.

To confirm the presence of HCV in the infected cells, total RNA from the cells was amplified by PCR methods, using probes with known HCV sequences. Details are given in Example 4. Figure 6 shows a gel analysis of the PCR 20 products from HCV-infected CU cell lines. Lanes 1-8 are CUL, CU3, CU4, CU5, CU6, CU8, CU9 and CU12, respectively. Lane 9 is a positive control of chimpanzee x198 liver RNA during the acute phase of HCV infection and was processed identically as the CU RNA samples. Lanes 10 and 11 are the 25 cDNA and PCR negative controls to demonstrate the lack of contamination during the PCR assay. Lane 12 is lambda DNA cleaved with HindIII as size markers. Lane 5 (CU6) and 9 (PCR positive control) show a positive reaction. All lanes have a lower band that represents the primers used in the 30 PCR reaction.

Figure 7 shows a gel analysis of PCR products from HCV-infected CHMP cell lines. Lanes 1-12 represent CHMP

1.21, 1.22, 1.23, 1.24, 1.25, 1.26, 1.27, 1.28, 1.29, 1.30, 1.31 and 1.32, respectively. Lane 13 is the PCR analysis of the inoculum used to infect both the CU and CHMP cell lines. Lane 14 is CHMP 2.02. Lane 15, 18 and 19 are PCR 5 positive controls. Lane 15 and 18 are xI98 liver RNA as described for Figure 6. Lanes 16 and 17 are cDNA and PCR negative controls, respectively. Lane 19 is a PCR positive control consisting of a gel purified band from a cloned fragment of HCV homologous to the PCR primers used in this 10 assay. Lane 20 is HindIII digested lambda DNA as size markers.

Positive reactions were obtained with CHMP 1.27 and 2.02, the inoculum used to infect the cell lines, and each of the positive controls. The negative controlled were 15 negative indicating that no contamination occurred during the PCR reaction.

Thus, of the 20 CU and CHMP cell lines tested, three have been shown to be permissive for infection with and replication of HCV. The cell lines are CHMP 1.27, CHMP 20 2.02 and CU6. These cell lines are selected as meeting both criteria of the cells of the present invention: (a) secretion of liver-specific proteins, and (b) ability to support hepatitis virus infection.

These results demonstrate that the immortalized cell 25 lines of the invention are infectable with HCV, and support replication of HCV, for use in the production of HCV for use in diagnostics and vaccines, and the use of the infected cell lines for testing antiviral compounds for the inhibition of HCV replication.

30 Infection of immortalized primate hepatocytes by HAV is demonstrated in the study reported in Example 7, which shows HAV infection and growth in immortalized marmoset

hepatocytes. After inoculating the cells with a HAV inoculum, total cellular RNA was isolated by the method as described above and the samples were analyzed by PCR, using probes with HAV-specific sequences. Figure 8 shows the gel analysis of PCR products from HCV-infected CJS1 cell line. Lane 1 is PCR negative control; lanes 2-5, uninfected CJS1 cells; lanes 6 and 7, HAV-infected CJS1 cell lines with 5% of the nucleic acid being examined by PCR; lanes 8 and 9, the HAV negative inoculum used for infection of CJS1 in lanes 4 and 5; lanes 10 and 11, PCR analysis of the inoculum for lanes 6 and 7; lane 12, weak PCR positive control; and lane 13, strong PCR positive control. The positive controls were positive, the negative controls were negative, the uninoculated cells were negative, the inoculated cells were positive, and the inoculum was positive. As seen, one of the cell lines, CJS1, was infectable by HAV.

It will be appreciated that the immortalized hepatocytes of the invention can be infected by other hepatotropic viruses, such as HBV, and enterically transmitted Non-A, Non-B hepatitis virus, for viral replication in the cells. The methods described above indicate that immortalized primate hepatocytes capable of supporting hepatitis viral replication in culture occur at a frequency which allows their selection from a relatively small number of possible cell lines.

III. Utility

A. Virus Particles Production

As demonstrated above, the immortalized cells of the present invention are infectable with and support replication of human hepatotropic viruses, such as hepatitis

viruses. The cells can therefore be used to produce such viruses in culture. As examples, immortalized chimpanzee or human hepatocytes can be used to produce HCV virus particles. A variety of primate hepatocytes, including 5 marmoset hepatocytes, can be used to produce HAV particles.

In a typical method, an immortalized hepatocyte cell line capable of supporting replication of the hepatotropic virus is selected, for example, by detection of viral RNA in the infected cells, as described above. The cell line 10 is infected with the virus, typically by inoculating the cells with plasma from humans infected with the virus of interest, also as detailed above. Virus infection of the cells can be confirmed by PCR methods for detecting virus-specific RNA, described in Example 3, or by showing that 15 the culture medium is itself infective for primary or immortalized hepatocytes.

Virus particles in the culture medium can be isolated by a variety of available methods. In one approach, detailed in the companion PCT application for "Purified 20 Non-A, Non-B Hepatitis Virus", culture medium harvested from the infected cells was first clarified by low-speed centrifugation ($12,000 \times g$ for 30 minutes), and the clarified material was layered over a discontinuous 20% sucrose and 68% sucrose layers, and centrifuged at high 25 speed ($131,000 \times g$) for 3.8 hours. The material at the 20%/60% interface is collected, diluted with buffer, then centrifuges a second time over a 68% sucrose cushion at 154,000 $\times g$ for 16 hours. The material at the interface contains the desired HCV particles, as demonstrated by PCR 30 methods and electron microscopic analysis. Similar centrifugation methods may be applied to obtain other

hepatitis virus particles in purified or partially purified form.

Alternatively, the virus particles may be isolated from the culture supernatant by affinity chromatography 5 methods, using immobilized anti-virus antibodies, such as monoclonal antibodies prepared against the virus particles, to capture the particles on a solid support.

The isolated virus particles can be used, in attenuated or inactivated form, in a vaccine composition, or as a 10 diagnostic reagent. The virus particles may alternatively be subfractionated into component mature viral proteins, for use in a vaccine composition or as a diagnostic reagent.

15 B. Drug Screening

Immortalized cells which are infected with a selected hepatotropic virus, in accordance with the invention, may be employed in a drug screening method, for identifying drug compounds effective to inhibit the growth of the virus 20 in hepatocytes. In this method, a culture of infected cells are exposed to the test compound, typically over a selected drug concentration range, for a given inhibition period, typically 6-48 hours. Thereafter, the cells, or virus particle obtained from the cells, are examined for 25 level of virus.

One general method for quantitating virus levels in infected cells is described in Example 3, for detection of HCV infection in immortalized chimpanzee hepatocytes. Here total RNA is isolated from infected cells, for amplification by standard polymerase chain reaction (PCR) methods, 30 employing known, virus-specific sequences. The level of

RNA can be quantitated, for example, by dot-blot hybridization (Sambrook).

Virus levels can be quantitated by measuring the levels of virus-specific cell-surface antigens. U.S.

5 Patent No. 4,777,245 describes an IgM monoclonal antibody which is specific against cell surface antigen in HCV-infected liver cells. The level of antibody binding to the cells can be quantitated, for example, by a standard enzyme immunoassay methods employing an enzyme-labeled antibody
10 specific against the first-bound antibody.

From the measured inhibition of virus growth in infected, treated cells, compounds which are likely candidates for in vivo testing can be identified.

15 C. Secretory Protein Production

One of the important features of the immortalized cell lines of the invention is the ability to synthesize liver-specific secretory proteins, such as albumin, α -1-antitrypsin, complement C'4, fibrinogen, apolipoproteins A-1 and E, 20 transferrin, and plasminogen. As noted above, there is some variation in the type and levels of various serum proteins which are synthesized by different immortalized cell lines. This suggests that immortalized cells with highly specific protein-synthesis specificity can be
25 identified, for use in production of selected serum proteins.

The immortalized cells are grown on a suitable growth medium, such as the SFM medium described above, which maintains the differentiated state of the cells. The
30 selected secretory proteins are obtained from culture medium, and isolated from the medium by standard protein fractionation methods.

The following examples illustrate specific methods for producing immortalized primate hepatocytes, and for characterizing the morphology, cell products and viral infectivity of the cells. The examples are intended to 5 illustrate, but not limit, the scope of the invention.

Example 1

Primary Hepatocyte Cells

A. Cultured Primary Chimpanzee Hepatocytes

10 Liver cells were obtained from a five-year-old male chimpanzee (*Pan troglodytes*) (Joshua (PTTx266), housed at a primate facility. The animal had not been previously exposed to hepatitis A virus, hepatitis B virus, delta hepatitis virus, non-A, non-B hepatitis virus, or human 15 immunodeficiency virus. Liver wedge biopsy was performed as described by Eichberg. Hepatocytes were isolated by standard perfusion and collagenase treatment of the liver wedge as has been described (Lanford, 1989).

In this and the other examples below, the serum-free 20 media (SFM) formulation utilized a basal medium supplemented with 10 mM HEPES, pH 7.4, 2.75 mg/ml NaHCO₃, and 50 µg/ml gentamicin, together with the supplements as listed below. In the described media of Table 3, Williams Medium E (WME) served as a basal medium. Although WME is presently 25 preferred as the basal medium of the serum-free medium other commercial media formulations can be expected to give satisfactory results. For instance, a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Salas-Prato) or RPMI 1640 (Gibco) (Enat, Sell) should give 30 satisfactory results when supplemented with the supplements listed in Table 3.

Table 3

<u>Supplement</u>	<u>Medium Concentration</u>
EGF	100 ng/ml
5 Insulin	10 μ g/ml
Glucagon	4 μ g/ml
BSA	0.5 mg/ml
Linoleic Acid	5 μ g/ml
Hydrocortisone	10^{-6} M
10 Selenium	10^{-8} M
Cholera Toxin	2 ng/ml
LGF	20 ng/ml
Transferrin	5 μ g/ml
Ethanolamine	10^{-6} M
15 Prolactin	100 ng/ml
Somatotropin	1 μ g/ml
TRF	10^{-6} M

To prepare the media, the supplements were added in
 20 the following quantities in Table 3 to 500 ml of WME in a
 sterile plastic bottle:

5 ml 50 mg/ml BSA (bovine serum albumin), 500 μ g/ml
 Linoleic Acid
 25 0.5 ml 5 mg/ml Insulin
 0.5 ml 5 mg/ml Insulin,
 5 mg/ml Transferrin, and 5 μ g/ml
 Selenium (ITS)
 50 μ l 10^{-2} M Hydrocortisone
 30 5 μ l 200 μ g/ml Cholera toxin
 0.5 ml 100 μ g/ml EGF (epidermal growth factor)

50 μ l 10⁻² M Ethanolamine
0.5 ml 1 mg/ml Somatotropin
50 μ l 1 mg/ml Prolactin
0.5 ml 10⁻³ M Thyrotropin Releasing Hormone
5 50 μ l 200 μ g/ml LGF (liver growth factor, i.e., glycyl-histidyl-lysine)
1 ml 2.0 mg/ml Glucagon
WME was purchased with L-glutamine and without NaHCO₃ from Hazelton Research Products, Inc. (Denver, Pennsylvania). Supplements, including growth factors and hormones were obtained from Sigma (St. Louis, MO) or Collaborative Research (Bedford, MA).

Dissociation of primary cells and subsequent passages utilized a collagenase/dispase (Boehringer Mannheim) solution at a concentration of 100 μ g/ml in phosphate-buffered saline (PBS, pH 7.2). Following dissociation, a five-fold excess of 5% fetal bovine serum in Williams medium E (5% FBS/WME) was added to the solution. Cells were pelleted at 50 x g for six minutes, resuspended in a minimal volume of 5% FBS/WME and allowed to attach during a 2-3 hour period at 37°C, 10% CO₂. Cells were maintained with SFM and changed at two-day intervals.

Under the culture conditions described, the primary cells maintain the characteristics of highly differentiated hepatocytes, as evidenced by cell morphology, and the ability to produce and secrete several liver-specific secretory proteins, including albumin, α -1-antitrypsin, complement C'4, fibrinogen, apolipoproteins A-1 and E, transferrin, and plasminogen. The characteristics and stability of the primary cultured cells have been reported by the inventors (Lanford, 1989).

Example 2Immortalizing Chimpanzee Primary Hepatocytes

The U19-5 cell line which constitutively produces the U19 amphoteric retrovirus was a gift from Drs. P.S. Jat and 5 P.A. Sharp, M.I.T. (Cambridge, MA). The retrovirus construct has been described in detail (Jat). The construct produces a large T antigen protein defective for binding to the SV40 origin of DNA replication.

The U19-5 cell line was grown in DMEM medium (Dulbec-10 co's modified minimal medium, available from Gibco, Grand Island, NY) with 10% FBS (fetal bovine serum) under standard culture conditions (Jat, 1986). Culture medium was collected at 24-hour intervals and passed through a 0.45 μm filter (Amicon, Beverly, MA) prior to use for 15 infection of primary hepatocyte cultures.

Subconfluent cultures of primary hepatocytes (Example 1) were infected one day post-plating by the addition of 1 ml of U19-5 culture medium to the cells in the presence of PolybreneTM (8 $\mu\text{g}/\text{ml}$). The plating density was such as to 20 allow the cells several rounds of cell division to occur after introduction of the oncogene. After incubation overnight, cells were washed three times with WME and maintained in SFM until colony outgrowths were observed, typically about 1 month after infection.

25 The cells were selected for G418 resistance by addition to the culture medium of G418 (Geneticin, GIBCO) (400 $\mu\text{g}/\text{ml}$). The cells were then treated by a collagenase-disperse (Boehringer Mannheim) solution at a concentration of 100 $\mu\text{g}/\text{ml}$ in phosphate-buffered saline (PBS, pH7.2) for 30 10 minutes at 37 °C. Following dissociation, a five-fold excess of 5% fetal bovine serum in Williams medium E (5% FBS/WME) was added to the solution. Cells were pelleted at

50 x g for six minutes, resuspended in a minimal volume of 5% FBS/WME and allowed to attach during a 2-3 hour period at 37°C under 10% CO₂. The cells were plated at a low cell density so that single colony outgrowths could be isolated and subcloned. From over 100 colonies, over 70 were picked based upon differences in morphological appearance. The cells are designated CHMP cells, and are assigned cell line numbers, such as CHMP 1.21, CHMP 1.22, etc.

10

Example 3CHMP Cell Properties

A. Morphological Characteristics

Immortalized cell lines with several different were 15 morphologies were observed. Four dominant morphologies are seen in the photomicrographs (225 magnification) in Figures 1A-1D, as described above.

B. Secretory Proteins

20 Hepatocyte cultures representing compact cell morphology (CHMP 5.13, CHMP 1.05, CHMP 2.01, CHMP 2.06, and CHMP 3.11), flat cell morphology (CHMP 5.13), and cuboidal cell morphology (CHMP 5.04) were incubated with 100-250 uCi [³⁵S]-methionine (.800 Ci/mmol, ICN, Costa Mesa, CA) for 48 25 hours. A portion of the culture medium from each cell line was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 0.1% SDS, 12% acrylamide, according to standard procedures (Laemmli). The gels were developed at -70°C on Kodak XAR-5 film for 2 days, with the 30 results shown in Figure 3. The major proteins detected by this analysis had molecular sizes corresponding to albumin (67kd) and alpha-1-antitrypsin (54kd). The size markers

(molecular weight given in kilodaltons) were phosphorylase B (93kd), serum albumin (67kd), ovalbumin (43kd), carbonic anhydrase (30kd), trypsin inhibitor (20kd), and lysozyme (14kd).

5 In another study, proteins secreted by normal chimpanzee primary hepatocytes (Example 1) and the CHMP 1.20 cell line were compared by immunoprecipitation from labeled culture medium. Antibodies against human serum albumin, α -1 antitrypsin, apolipoproteins A-1 and E, β -2 microglobulin, 10 complement C'4, C-reactive protein, fibrinogen, prealbumin, plasminogen, and transferrin, were obtained from Calbiochem, San Diego, CA).

15 Immunoprecipitation was carried out according to published procedures (Jacob, 1989). Briefly, the labeled medium was incubated 16 hr at 4°C with antibodies specific to the various plasma proteins bound to protein A agarose beads (BRL, Gaithersburg, MD). The beads were washed three times to remove unbound proteins and the bound proteins were eluted with SDS-gel electrophoresis sample buffer. 20 The samples were analyzed by 12% SDS-PAGE and fluorography, as described above. The identity of the immunoprecipitated proteins are indicated at the top of the figure. The profile of secretory proteins obtained with primary hepatocytes (Figure 4A) was very similar to that observed 25 for the CHMP 1.20 cell line (Figure 4B), demonstrating the retention of liver-specific functions, i.e., a highly differentiated state, after immortalization.

In a third study, CHMP cell lines 1.20, 1.33, 2,02, 2.03, 2.05, 3.01, 4.03, 4.07, 5.01 were cultured in the 30 presence of 35 S-methionine, as above, for 48 hours when the cultures were 80-90% confluent. Culture medium from each cell line was immunoprecipitated with the immobilized anti-

human serum protein antibodies noted above, plus an antibody specific against α -fetoprotein. The immunoprecipitated proteins were separated by SDS-PAGE and the gels developed by autoradiography. Table 2 above summarizes the plasma 5 proteins expressed from the representative CHMP cell lines compared with that observed in normal primary cultures. The "+" symbol in the table means normal expression, i.e., expression comparable to that observed in primary cultures; the "*" symbol, low expression; the "--" symbol, no 10 expression; and "NT", not tested.

C. Oncogene Expression

Each of CHMP cell lines 1.20, 1.33, 2.02, 2.03, 2.05, 3.01, 4.03, 4.07, 5.01 was also examined for the presence of integrated U19 provirus T antigen/neomycin genes. This 15 was done by digestion of total cellular nucleic acids with the restriction enzyme BamHI, under standard digest conditions (Maniatis). This digestion releases the SV40 T antigen gene which is placed between BamHI sites in the retroviral plasmid construct. The number of proviral 20 inserts can be determined by hybridization to the neomycin gene, since one BamHI site is contributed by flanking cellular sequences. Digested nucleic acids were separated by electrophoresis on 1% native agarose gels. After capillary transfer to nitrocellulose membranes, filters 25 were baked at 80°C for two hours. Hybridization to the neomycin gene was performed at 65°C in a 0.01% Denhardts solution (Denhardt) containing 1% SDS and 1M NaCl. The plasmid construct pSV3neo, which contains the SV40 T antigen and neomycin gene sequences, was nick-translated 30 and used as the hybridization probe. The methods follow conventional techniques (Sambrook, Southern). Hybridiza-

tion to the nick-translated pSV3neo probe was observed in each of the cell lines.

In addition, each of the cell lines was examined for the presence of T antigen in 35 -methionine labeled cell 5 lysates, using immunoprecipitation with immobilized anti-T antigen antibody. As shown at the right in Table 3, T antigen was present in each of the immortalized cell lines.

Example 4

10 HCV Infection of CHMP Cells

A. Viral Inoculation

Immortalized chimpanzee hepatocytes derived from HCV-infected primary hepatocytes were prepared substantially as 15 described in Example 2, but using hepatocytes obtained from a liver biopsy of a chimpanzee during acute-phase HCV infection. The cell lines are designated CU cell lines.

Several CHMP and CU cell lines were cultivated on collagen coated 25 cm² Primaria flasks in SFM under normal 20 conditions (37 °C, 10% CO₂ atmosphere). When the cultures reached a level of 90% confluence, they were inoculated with chimpanzee plasma known to contain HCV.

The inoculum was a pool of plasmas obtained from three chimpanzees (x7, x268, and xl74) during the acute phase of 25 a HCV infection and did not contain any other infectious agent. The plasmas were diluted 5-fold in SFM and 1 ml was added to the cultures. After incubation for 3 hr at 37°C, another 3 ml of SFM was added to the cultures and the incubation was continued for 16 hr. The cultures were 30 washed three times with WME to remove the inoculum and SFM was added. The medium was changed every other day and on

the 11th day after infection the cultures were harvested for analysis.

B. RNA Characterization

5 The cells were washed three times with phosphate buffered saline (PBS) and the cellular RNA was extracted and purified using a standard GITC extraction procedure (Chomozynski). The cells were lysed with a solution containing 4M guanidine isothiocyanate, 0.18% 2- mercapto-
10 ethanol, and 0.5% sarcosyl. The cell lysate was extracted several times with acidic phenol-chloroform- isoamyl alcohol, and the RNA was precipitated with isopropanol. The purified RNA was resuspended in water and one tenth of each sample was used for polymerase chain reaction (PCR)
15 amplification to detect the HCV RNA genome.

PCR was conducted using standard methodology (Innis). The first step involved a cDNA reaction in which a DNA copy of the HCV RNA was made using reverse transcriptase and an oligonucleotide primer designated 6A that is complementary
20 to the strain of HCV used in our studies. The four primers used for cDNA and PCR were derived from the putative nonstructural region of HCV designated NS3 and their sequences are given below.

Primers:

25 5A 5' TCTGTGATAGACTGCAACACG 3'
6A 5' TTTGGTGATTGGGTGCGTCAG 3'
5B 5' GATGCTGTCTCCAGGACTCAA 3'
6B 5' AACAGCGCCCAGTCTGTATAGCAG 3'

The sequence of these primers was derived from the
30 sequence of a cDNA clone of a strain of HCV as previously described (Jacob). A portion of the cDNA reaction mixture (1/4th) was PCR amplified for 35 cycles using the Taq

polymerase and the oligonucleotide primers 5A and 6A. A portion of the first round of PCR (1/50th) was used for a second round of PCR using the primers 5B and 6B.

Figure 6 shows a gel analysis of the PCR products from 5 HCV-infected CU cell lines. Figure 7 shows a gel analysis of PCR products from HCV-infected CHMP cell lines. The gel results are discussed above.

Example 5

10 Immortalized Baboon Hepatocytes

A. Immortalization Method

Plasmid p1Aneo which contains the adenovirus E1A oncogene was obtained from Drs. E. Ruley and K. Maruyana, Cancer Research, Department of Biology, Massachusetts 15 Institute of Technology. The plasmid construction has been described (Van de Woude). Plasmid pSVC myc-1 that contains the myc oncogene was obtained from the American Type Culture Collection (Rocklawn, MD), and is identified by ATCC No. 41029. Plasmid pUJ EJ 6.6 that contains the ras 20 oncogene was also obtained from the American Type Culture Collection, and is identified by ATCC No. 41028.

Primary baboon hepatocytes were prepared from a 2 year old female baboon housed at the Southwest Foundation for Biomedical Research (San Antonio, TX). Hepatocyte cells 25 were obtained from a liver wedge biopsy and cultured to form a stable primary hepatocyte cell line in SFM, as detailed in Example 1. The primary hepatocyte cells retained liver-specific function for several months in culture, as judged by the ability to secrete liver-specific 30 proteins.

U19 retrovirus or plasmid(s) containing selected oncogenes were introduced into the cells by retrovirus

infection, in the case of the U19 retrovirus, or by lipofection, in the case of plasmids, according to the manufacturer's protocol (BRL, Gaithersburg, MD). Briefly, lipofection was performed by combining 5 μ g of plasmid DNA 5 for each plasmid, such as 5 μ g of pSVC myc-1 and 5 μ g of pAneo plasmid, in 1.5 ml of serum-free medium (SFM) with 30 μ g of LipofectinTM in 1.5 ml of SFM and adding to a 60 mm culture of subconfluent baboon hepatocytes. The mixture was incubated with the cells for 6-16 hours and the medium 10 then removed by washing. The cells were maintained in SFM for 7-10 days and then G418 (100-400 μ g/ml) for 2 weeks in SFM. Single colonies were then treated by a collagenase/dispase (Boehringer Mannheim) solution at a concentration of 100 μ g/ml in PBS, pH 7.2 for 10 minutes at 37°C. 15 Following dissociation, a five-fold excess of 5% fetal bovine serum in Williams medium E (5% FBS/WME) was added to the solution. Cells were pelleted at 50 x g for six minutes, resuspended in a minimal volume of 5% FBS/WME and allowed to attach during a 2-3 hour period at 37°C under 10% 20 CO₂. Cell lines established from single colony outgrowths and representing various oncogenes used to immortalize the primary hepatocytes were selected. The cells are designated BH cells, and are assigned cell line numbers, such as BH1A, BH3A, etc.

25

B. Cell Morphology

Phase contrast photomicrographs were taken of the living cell lines growing on collagen coated plastic surfaces, and representative cell types are shown in 30 Figures 2A-2D. The different morphologies represent those obtained when different oncogenes are used to immortalize baboon hepatocyte. Figure 2A shows cell line BH1A immor-

talized with the UI9 retrovirus; Figure 2B, cell line BH6A immortalized with a plasmid encoding both SV40 large and small T antigens, pSV3neo; Figure 2C, cell line BH5A immortalized with plasmids encoding both the myc and ras oncogenes; and Figure 2D, cell line BH3A immortalized with plasmids encoding both the myc and E1A oncogenes.

C. Secretory Proteins

Immortalized baboon cell line BH3A was examined for secretory proteins, substantially according to the method described in Example 2. Briefly, a 25 cm² flask of BH3A cells were labeled for 24 hours with ³⁵S-methionine and the labeled medium was harvested and clarified. An aliquot of the clarified medium was analyzed directly by SDS-PAGE. For immunoprecipitations, 300 µl of the labeled medium was incubated for 16 hours with protein A agarose beads containing bound IgG antibodies against specific liver proteins, as described in Example 2. After three washes to remove unbound protein, the proteins were eluted from the beads with SDS gel-electrophoresis buffer, and the eluted material was fractioned by SDS-PAGE and analyzed by autoradiography. Figure 5 shows the pattern of liver secretory proteins produced by the cell line, where the abbreviations at the top of the figure are for apolipoproteins E and A1, prealbumin, β_2 -microglobin, C-reactive protein, complement C'4, transferrin, α -1-antitrypsin, and albumin. It is seen from the figure that the BH3A cell line produces all of the liver-specific proteins except c-reactive protein.

Example 6Immortalized Marmoset Hepatocytes

A liver wedge biopsy was performed on a marmoset. A liver wedge of approximately 5 gm was perfused with 5 collagenase, using standard methods. Viable hepatocytes were plated on collagen-coated Primaria plates (Falcon, Lincoln Park, NJ). The primary hepatocytes were maintained in SFM medium as described in Example 1.

The hepatocytes were immortalized by the U19 retrovirus, using the procedures described in Example 2. Several of the cell lines, including one designated CJS1, were shown to retain their differentiated liver functions, as evidenced by secretion into the cell medium of several liver-specific proteins.

15

Example 7HAV Infectivity of Immortalized Marmoset Cells Line

The immortalized marmoset hepatocyte cell line CJS1 was grown on collagen coated Primaria 25cm² flasks in SFM as 20 described above. When the cultures reached 90% confluence they were infected with the HM175 strain of HAV (obtained from Dr. Mary Estes, Baylor College of Medicine, Houston, TX). The virus stock was diluted five-fold in SFM and added to the cultures. The cultures were incubated for 3 25 hr at 37°C with the inoculum, and then 1.5 ml of SFM was added to the cultures and the incubation was continued for 16 hr. The cultures were washed three times with WME to remove residual inoculum and changed to SFM. The medium was changed every other day and the cultures were harvested on 30 day 10 post-infection.

Total cellular RNA was isolated by the method described above and the samples were shipped to Dr. Estes

laboratory for PCR analysis. PCR was performed as described above on 5% of the RNA sample, and the PCR products were analyzed on a 1.5% seakem agarose gel. Figure 8 shows the gel analysis of PCR products from HAV-infected CJS1 5 cell line, as discussed above.

Although the invention has been described with respect to particular cell lines and infective agents, it will be apparent that hepatocytes from other primate species, such 10 as from humans, and other hepatotropic viruses, such as hepatitis B can be employed in the invention.

IT IS CLAIMED:

1. An immortalized primate hepatocyte cell line whose cells are characterized by:

5 (a) an oncogene integrated in the cellular genome;
 (b) secretion in culture of at least three hepatocyte secretory proteins selected from the group consisting of albumin, α -1-antitrypsin, complement C'4, fibrinogen, apolipoproteins A-1 and E, transferrin, and plasminogen; 10 and
10 (c) ability to support replication by human hepatotropic viruses.

2. The cell line of claim 1, wherein the relative 15 proportion of such secretory proteins produced by the cells is similar to that found in primate serum.

3. The cell line of claim 2, wherein the cells secrete albumin, α -1 antitrypsin, apolipoprotein-E, and 20 fibrinogen.

4. The cell line of claim 3, which is derived from primary chimpanzee hepatocytes, and the cells in the cell line are infectable with and support replication of 25 hepatitis C virus.

5. The cell line of claim 1, which is derived from a primary human or chimpanzee hepatocytes, and the cells in the cell line are infectable with and support replication 30 of hepatitis C virus.

6. The cell line of claim 1, which is infectable with and supports replication of hepatitis B or hepatitis A viruses.

5 7. The cell line of claim 1, wherein the oncogene is selected from the group consisting of SV40 large or small T antigen, adenovirus E1A, myc, ras, or combinations of these.

10 8. The cell line of claim 1, wherein the cell line is derived from chimpanzee or human primary hepatocytes, and the oncogene is an SV40 large T antigen.

15 9. The cell line of claim 1, wherein the cell line is derived from baboon primary hepatocytes, and the oncogene is a combination of E1A and myc oncogenes.

10. A method of producing hepatotropic virus particles, comprising

20 culturing the immortalized primate hepatocyte cell line of claim 1, after cell infection with the hepatotropic virus, and

harvesting the virus particles from the culture medium.

25

11. The method of claim 10, wherein the cell line is derived from human or chimpanzee hepatocytes, and the virus is hepatitis C virus (HCV).

30 12. The method of claim 11, wherein the cells are infected with HCV by exposing the cells in culture to chimpanzee or human HCV-infected serum.

13. The method of claim 10, wherein the cell line is derived from chimpanzee or human primary hepatocytes, and the oncogene is an SV40 large T antigen, and the virus is hepatitis C virus.

14. The method of claim 10, wherein said culturing is carried out in a serum-free medium which allows growth of the cell line without loss of liver-specific functions.

10

15. A method of screening compounds for ability to inhibit growth of an hepatotropic virus, comprising culturing the immortalized primate hepatocyte cell line of claim 1, after cell infection with the hepatotropic virus,
15 exposing the infected cells to the compound for a selected period, and assaying the cells for inhibition of virus growth.

20 16. The method of claim 15, wherein said assaying includes detecting the level of virus-specific nucleic acid present in the cell culture.

17. The method of claim 16, wherein the cell line is derived from human or chimpanzee hepatocytes, and the virus is hepatitis C virus (HCV).

25 18. The method of claim 17, wherein the cells are infected with HCV by exposing the cells in culture to chimpanzee or human HCV-infected serum.

19. A method of producing a liver secretory protein selected from the group consisting of albumin, α -1-antitrypsin, complement C'4, fibrinogen, apolipoproteins A-1 and E, transferrin, and plasminogen, comprising

5 culturing the cell line of claim 1 in a culture medium capable of maintaining the cells in the cell line in substantially differentiated state, and
 isolating the protein from the culture medium.

10 20. The method of claim 19, wherein the protein is fibrinogen or plasminogen.

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FIG. IA



B

FIG. IB



C

FIG. IC



D

FIG. ID



A

FIG. 2A



B

FIG. 2B



C



D

FIG. 2C

FIG. 2D

1 2 3 4 5 6 7 8 9 10 11 12

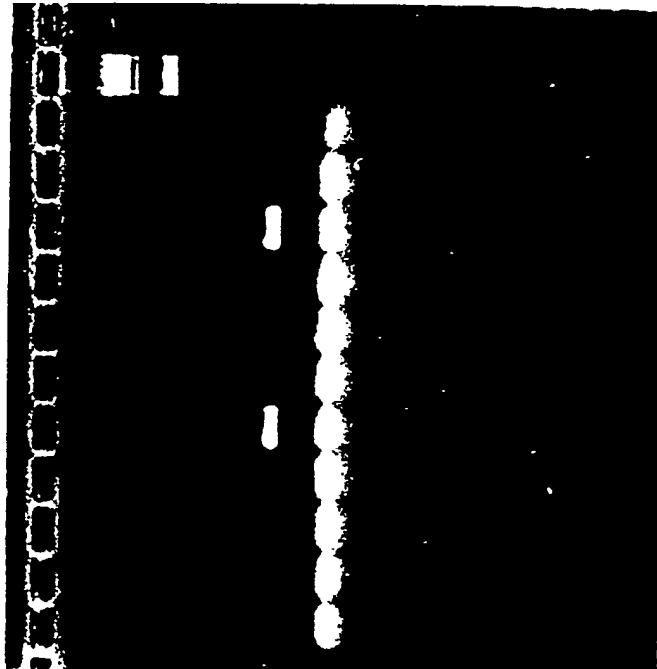


FIG. 6

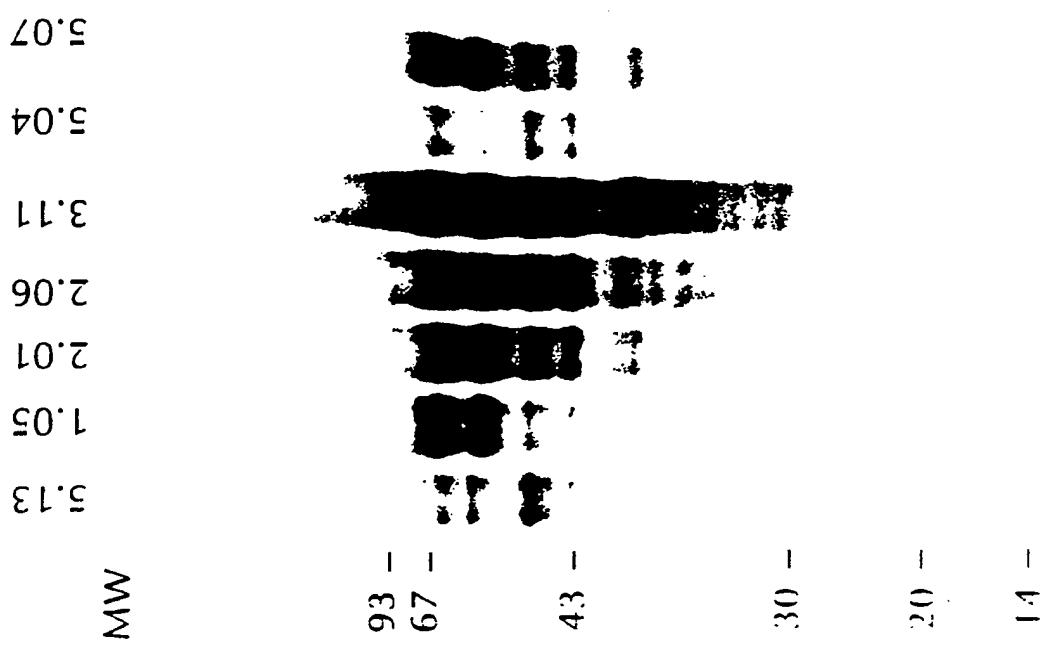


FIG. 3

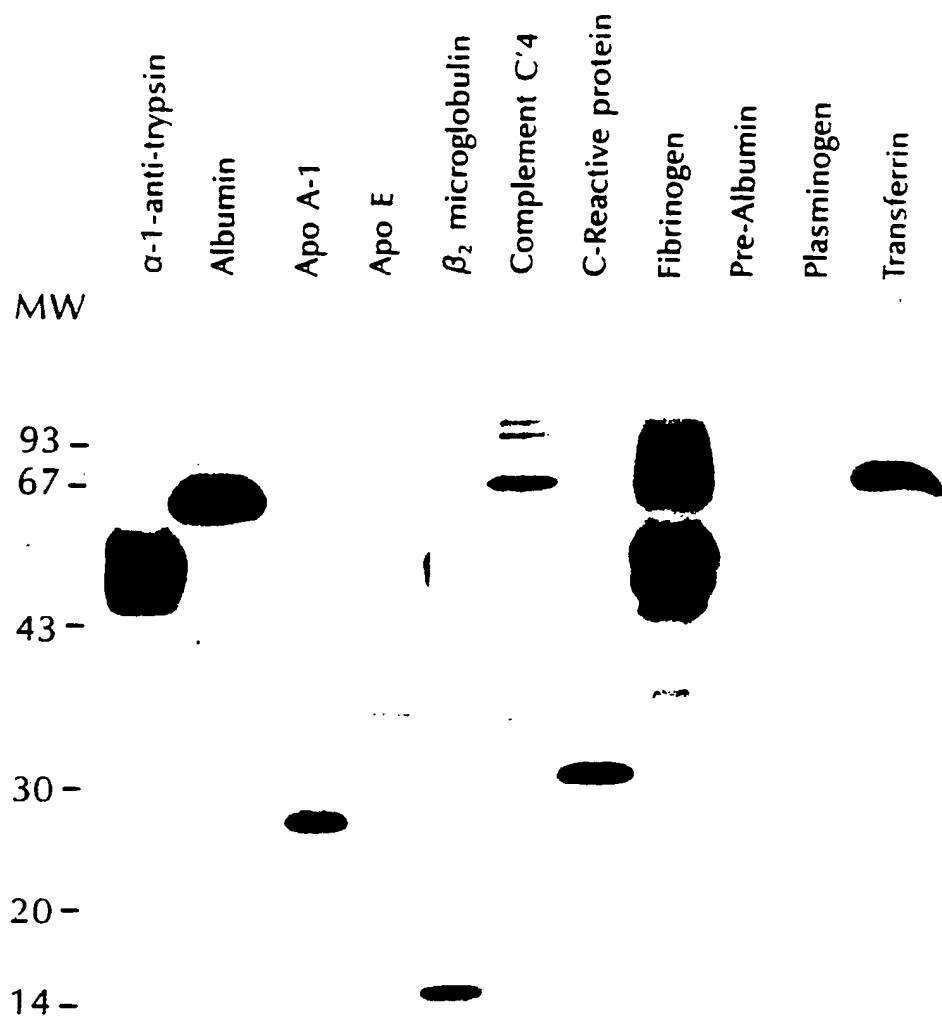


FIG. 4A

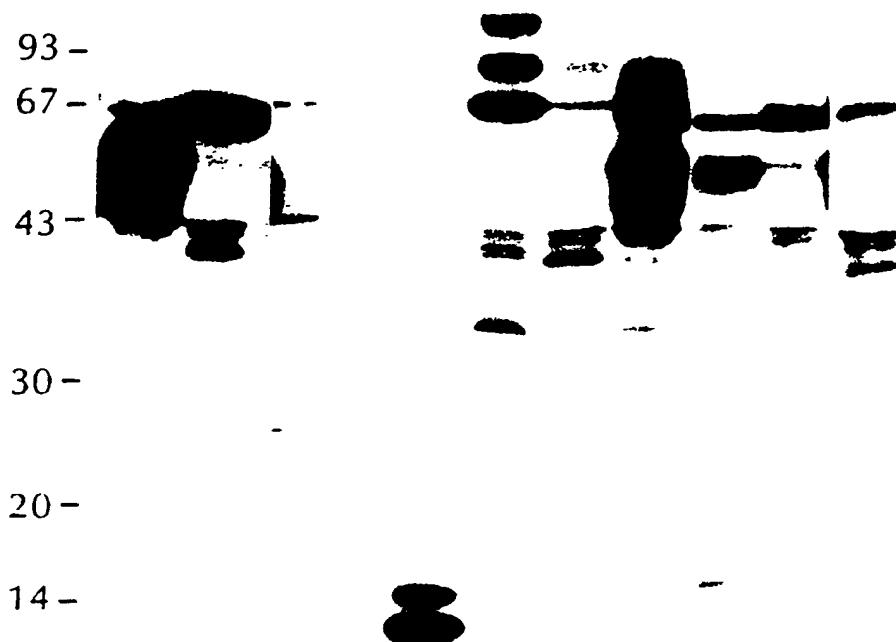


FIG. 4B

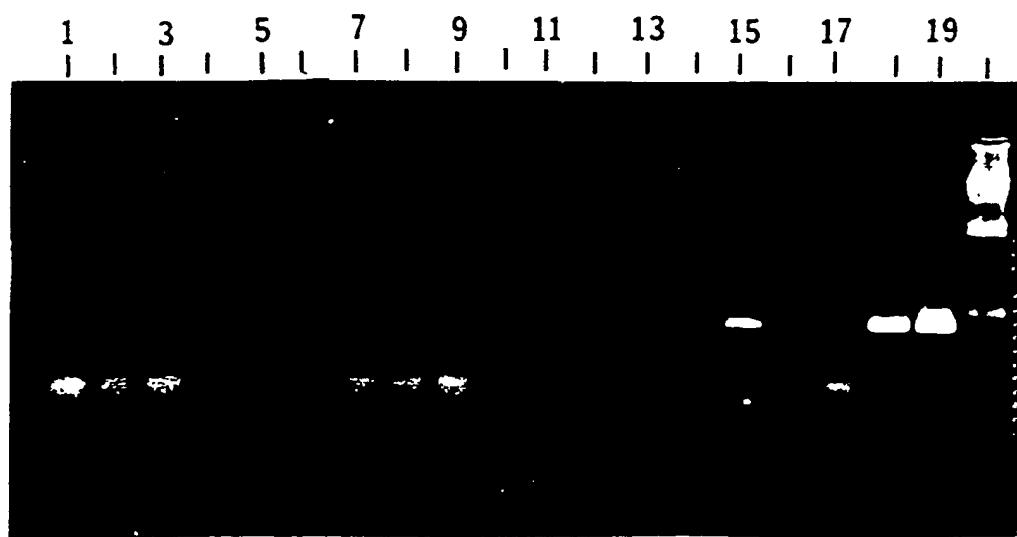


FIG. 7

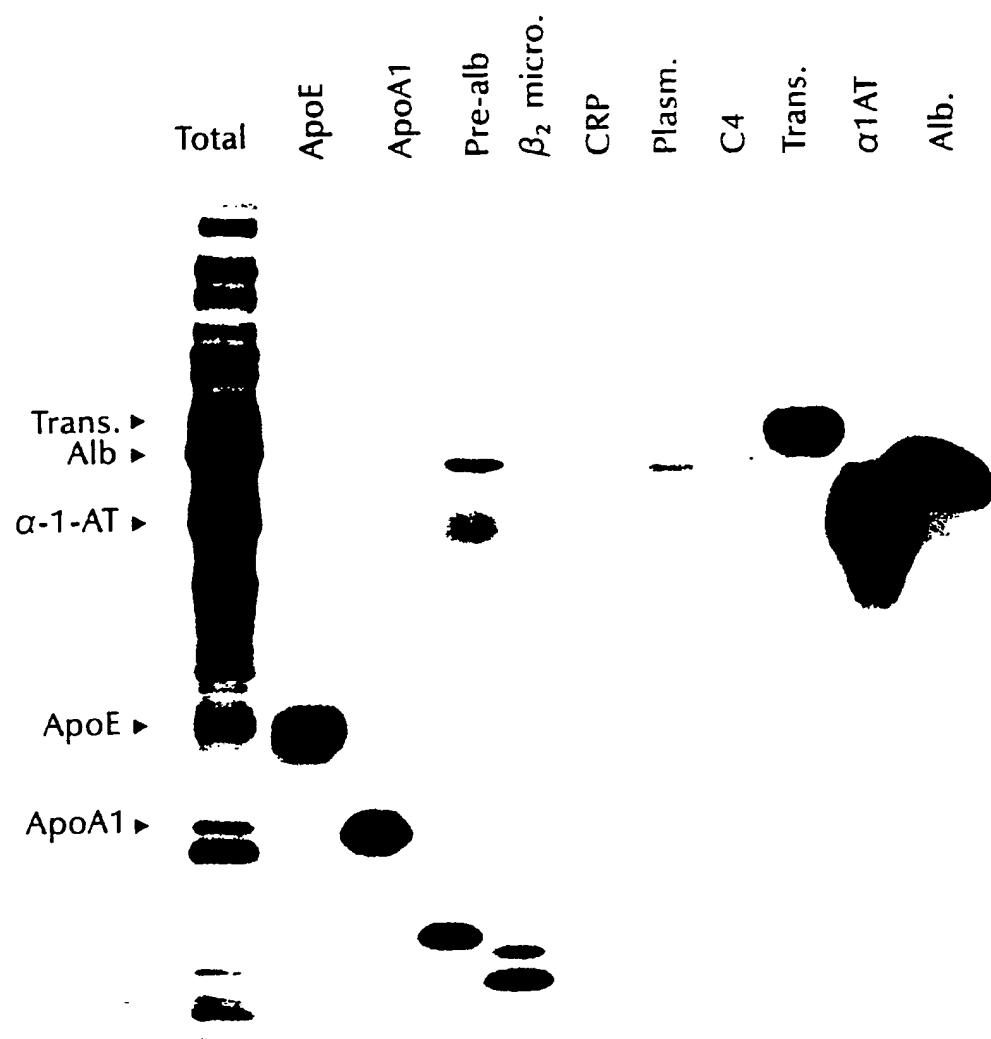
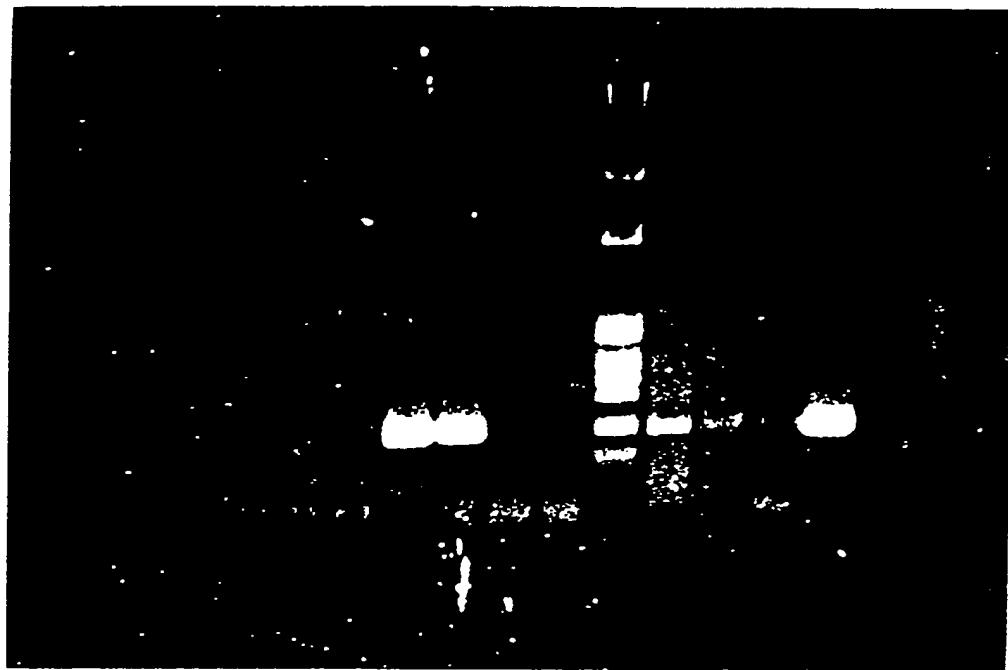


FIG. 5



1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 8

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/02296

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁵: C 12 N 5/10, C 12 P 21/22

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC ⁵	C 12 N
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸	

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	US, A, 7284368 (DEPARTMENT OF HEALTH AND HUMAN SERVICES) 29 August 1989 see the whole document --	1-8, 10, 14- 16, 19-20
A	Molecular and Cellular Biology, vol. 8, no. 10, October 1988, American Society for Microbiology, C.D. Woodworth et al.: "Tumorigenicity of simian virus 40-hepatocyte cell lines: effect of in vitro and in vivo passage on expression of liver-specific genes and oncogenes", pages 4492-4501 see the whole article --	1-20
A	Proceedings of the National Academy of Sciences, USA, vol. 81, October 1984, H.C. Isom et al.: "Quantitative assay for albumin-producing liver cells after simian virus 40 transformation of rat hepatocytes maintained in chemically defined medium", pages 6378-6382 see the whole article -----	1-20

* Special categories of cited documents: ¹⁰

- "A" document defining the general state of the art which is not considered to be of particular relevance
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

9th July 1991

Date of Mailing of this International Search Report

29. NR 91

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

W. J. Lenzen
Mme. M. van der Drift

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9102296
SA 46955

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 22/08/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 7284368	-----	None	-----